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Research Article

Antibiotics Susceptibilities and Plasmid Profiles of *Salmonella typhi* from Patients Attending Yobe State Specialist Hospital, Damaturu, Yobe State, Nigeria

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ABSTRACT

Salmonella infection remains a major public health concern worldwide. This study aimed to determine the antibiotics susceptibilities and plasmid profiles of *Salmonella typhi* from patients attending Yobe State Specialist Hospital, Damaturu, Yobe State, Nigeria. Two hundred and thirty three (233) samples (100 blood and 133 stool) were collected aseptically from patients diagnosed with enteric fever. The samples were processed using standard microbiological methods for identification of *Salmonella typhi.* The antimicrobial susceptibility patterns were determined using Kirby-Bauer disc diffusion techniques using ten antibiotics and the phenotypic expression of extended spectrum beta-lactamases (ESBLs) were determined using double disc diffusion test, and were further screened for plasmid DNA by standard method. Out of two hundred and thirty three (233) samples, thirty five isolates tested positive for *S. typhi* comprising of 20 (57%) from blood and 15 (43%) from stool. The stool Isolates were highly (100%) resistant to tarivid and less (13.3%) resistance to pefloxacin while blood isolates showed highest (60%) resistant against chlorophenicol and least (20%) resistance to cefriaxone fourteen (14) *Salmonella typhi* showed ESBLs production; the Stool samples has the highest (n=8, 57.1%) ESBLs producers while least (n=6, 42.8%) were from the blood samples. plasmid size and number determine by bands were separated using agarose gel electrophoresis. This study reveals that both the resistant antibiogram and plasmid profile are still viable epidemiological tools for tracing the sources of *Salmomella* isolates.

Keywords: *Blood; Stool; Salmonella typhi; Antimicrobial; Resistant; Plasmid*

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INTRODUCTION

The genus *Salmonella* belongs to the family Enterobacteriaceae. They are facultative anaerobic rods. *Salmonella* is mostly motile except *Salmonella pollurun* and *Salmonella galarum*, non-spore forming, Gram-negative bacterium (Salehi *et al*., 2015). There are currently over 2587 serotypes of *Salmonella,* responsible for salmonellosis, grouped into two basic species: *Salmonella enterica* and *Salmonella bongori* (Grimont *et al.,* 2007). *Salmonella* spp. is mainly transmitted by the faecal-oral route mainly through contaminated food or water. Outbreaks of *Salmonella* -associated diseases are usually associated with contaminated water and the ingestion of contaminated food of animal origin like, fishes, poultry, meat and milk (WHO, 2014).

Salmonella enterica is an important cause of human morbidity and mortality worldwide. *Salmonella typhi*

is the etiological agent of typhoid fever while Non-Typhoid *Salmonella* species (NTS) are associated with gastroenteritis and invasive infection in children, the elderly and immuno compromised patients; these include: *Salmonella Typhimurium, Salmonella enteritidis* amongst other serovars of *Salmonella enterica* that affect both human and animal, of which *S. Typhi* affects human only. Both *Salmonella typhi* and NTS are among the most frequent pathogens causing blood stream infections (BSI) in tropical low-resource settings (*Reddy et al.* 2010). The highest incidence and prevalence of *Salmonella* infection worldwide occur in Asia region (*Crump et al* .2013). Mainly in south and Southeast Asia, where isolates show high rate of antibiotic resistance. It is estimated that each year there are approximately 21.6 million cases of typhoid fever which result in 200,000 deaths worldwide (Adeshina *et al.,* 2010). However, the incidence of cases and death has been greatly increased by combination of poor sanitation and hygiene, unavailability of vaccines and high cost of effective antimicrobial chemotherapy (Adeshina *et al,* 2010).

In Nigeria, the antibiotics mostly readily available for treatment of typhoid and other *Salmonella* related diseases are chloramphenicol, ampicillin, tetracycline, third generation cephalosporin, gentamycin, quinolones and trimethoprim/sulfamethoxazole (El – Sayed *et al.,* 2012).

Recently multi-drug resistant (MDR) strains have emerged presumably due to the extensive use of antimicrobial both in human and animals' husbandry. In veterinary medicine for instance, antibiotics are being used in livestock production, disease prevention and as growth-promoting feed additives. The use of antibiotics in animals' production disrupts the normal floral of the intestine; prolongs faecal shedding of these organisms into the environment and emergence of antibiotics-resistant *Salmonella* strains. MDR in *Salmonella* is a cause of great concern in both clinical and veterinary medicine, as it may limit the therapeutic options available for the treatment of *Salmonella* -associated diseases (*Davis*, 2015).

This study aim to isolate, identify and plasmid profile of *Salmonella typhi* from blood and stool samples among patients attending Yobe State specialist hospital Damaturu, Yobe State.

MATERIALS AND METHODS

Study Area

The study was carried out in Yobe State Specialist Hospital (YSSH) Damaturu. Damaturu is the capital city of Yobe State, Nigeria, located in Northeast Nigeria. It's located on coordinates: 12°00'N 11°30'E/12.000°N 11.500°E). Yobe state is mainly an agricultural state, it was created on August 27, 1991 out of old Borno State. The state borders the Nigeria states of Bauchi, Borno, Gombe, and Jigawa while to the North of Niger Republic, it borders the Diffa Region and the Zinder Region.

Study Design, Sample Collection and Preparation

During a six months period (April-september, 2023), the sample populations were grouped into two with a total of 233 clinical samples of 100 blood samples, and 133 stool samples from patients attending the Yobe State Specialist Hospital (YSSH) Damaturu that shows symptoms of enteric fever. All patients were instructed on how to collect appropriate specimen.

Blood Culture

Using a sterile syringe and needle, 4ml of whole blood from the patient was collected and dispensed into 20ml tetrathionate. The mixture was incubated overnight at 37^0C for 18-24hrs in an incubator. Tubes that show turbidity were sub-cultured each from each of the containers unto freshly prepared sterile and dried *Salmonella*-Shigella agar (SSA), and incubated at 37^0C for 18-24 hours in an incubator. The sub culturing was done three times before conclusion that there is no growth (Sur *et al.,* 2007).

Subculture and Characterization

The pure isolates was obtained after the subculture and it was characterized using a gram stain and biochemical tests. The pure isolates were prevserved in a slant vijour bottle for next usages (Chessbriugh, 2007).

Stool Culture

The purulent or mucoid parts of the stool samples were picked using a sterile wire loop and inoculated into selenite F broth medium and then incubated at 37^0C overnight for 18 to 24hrs in an incubator. The broth cultures were subcultured in SSA. The SSA, and MCA plates were incubated overnight at 37^0C for 18 to 2hrs in an incubator (Sur *et al.,* 2007).

Antimicrobial Resistant Profiles

Antimicrobial resistance profile of *Salmonella* isolates were determined by the disc diffusion method of Kirby Bauer, (1966) and zones of inhibition interpretation was carried out as described by the

Clinical Laboratory Standard Institute (CLSI, 2014).The antibiotic disks used are manufactured by using the disc diffusion method of Kirby Bauer as described by the Clinical Laboratory Standard Institute (CLSI, 2016). Each *Salmonella* isolates was transferred in to Muller hilton broth and incubated at 37oc for 24 hours. The turbidity of the suspension was adjusted aseptically with sterile saline to obtained turbidity of 0.5 McFarland standards. Then, pour on muller hinton agar plate to cover their surface and then drained the excess media. The antibiotic disks were placed on the surface of agar at equal distance, sufficient to separate them from each other to avoid overlapping of the inhibition zones. Each plate carries a maximum of ten disc. After 30 seconds of pre-diffusion, the plates were incubated at 37oc for 24hours followed by the diameter of inhibition zones, measurement and the adjusted to the nearest rounded number. Atotal of 10 antimicrobial agents were used in this study namely: Ten (10) antimicrobial discs was be used in this study these include: chloramphenicol (30µg), ceftazidime (30µg), ciprofloxacin (30µg), cefotaxime (30µg), amoxicillin (30µg), augmentin (10µg), gentamycin (30µg), pefloxacin (30µg), tarvid (10µg) and ceftriaxone (30µg) (CLSI, 2016).

Determination of Extended Spectrum β-lactamase (ESBL) among the Isolates

This test was carried out by placing augmentin (AMC 30µg) antibiotics disc at the centre of sterile Mueller Hinton agar plate containing the streaked colonies of the positive isolate of *Salmonella typhi* on agar culture, ceftazidime (CAZ 30µg), cefotaxime (CTX 30µg) and ceftriaxone (CRO 30µg) were placed beside augmentin (AMC 30µg) disc with distances of 15mm from the centre and the plate was incubated overnight at 37^{o}C for 24 hours in an incubator. All clear zone of inhibition towards the Augmentin (AMC 30µg) showed positive results for the production of ESBL (CLSI, 2016).

Plasmid Curing of the Isolates

Single colonies of isolates were inoculated into nutrient broth supplemented with 2% SDS, 0.1% acridine orange (Electran) and incubated at 37°C for 24 h after which they were plated out on nutrient agar, incubated at 37°C for another 24 h (Durve *et al*., 2013). Colonies obtained were then harvested and subjected to plasmid extraction.

Plasmid Extraction of Salmonella Isolates

The DNA templates of each of the confirmed pure culture of *Salmonella typhi* isolates were generate by dispensing pure colonies of overnight grown culture unto 100-µL1X Tris- EDTA buffer, vortex mixed and boiled at 100°C for 10 minutes. Thereafter, transferred immediately to the freezer (-20°C) for 10 minutes, maintained at room temperature, vortex mixed again and centrifuged at 10,000 rpm for 10 minutes. The resulting supernatant containing DNA templates of the isolates were separated, stored at 4°C, and used as DNA template for PCR amplification (Yang *et al*., 2008).

DNA Quantification of Extracted DNA

The extracted genomic DNA was quantified using the Nano drop 1000 spectrophotometer. The software of the equipment was launched by double-clicking on the Nano drop icon. The equipment was initialized with 2 ul of sterile distilled water and blanked using normal saline. Thereafter, 2µl of the extracted DNA was loaded into the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the “measure” button (Abimiku *et al*., 2019).

Amplification of 16S rRNA Gene of the Isolates

The 16S rRNA genes of the *Salmonella* isolates were amplified using the universal primer 27F:5'-GGAACTGAGACACGGTCCAG-3’ and 1492R: 3'-CCAGGTAAGGTTCTTCGCGT-5’ (Zymo Research, USA) on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 µl for 35 cycles. The PCR mix included: X2 Dream Taq Master Mix (Taq polymerase, dNTPs, MgCl, Zymo research, USA), the primers at a concentration of 0.4 M and the extracted DNA as a template. The PCR conditions were as follows: 5 minutes at 95^{0}C for initial denaturation, 30 cycles each 1 minute at 94^{0}C for denaturation, 1 minute at 60^{0}C for annealing and 30 second at 72^{0}C for extension and final extension at 72^{0}C for 10 minute. The product was resolved on a 1% agarose gel and visualized on a UV transilluminator.

Detection of all the amplified genes by Agarose Gel Electrophoresis

One percent (1%) agarose gel was used to resolve DNA fragment. This was prepared by combining 1g agarose in ten times concentration of tris-borate ethylene diamine tetraacetate (10ml 10XTB-EDTA) buffer and 90ml sterile distilled water in 250ml beaker flask and heating in a microwave for 2 minutes until the agarose is dissolved. Exactly 0.7µl of ethidium bromide was added to the dissolved agarose solution with swirling to mix. The gel was then poured onto a mini horizontal gel electrophoresis tank and casting combs was inserted.

The gel was allowed to set for 30 minutes. The casting combs were carefully removed after the agarose gel had solidified completely. One times concentration (1X) TBE buffer was added to the reservoir until it covered the agarose gel. Precisely 8µl of gel tracking dye (bromophenol blue) was added to 10µl of each sample with gentle mixing. The sample was loaded onto the wells of the gel at a concentration of 10µl, the mini horizontal electrophoresis gel setup was covered and electrodes connected. Electrophoresis was carried out at 100-200mA for one hour. At the completion of electrophoresis, the gel was removed from the buffer and visualized under UV light and documented.

Conjugation Experiment

Salmonella isolates harbouring the group of ESBLs were selected for the conjugation experiment using the broth mating technique described by Chen *et al.*, (2007) with rifampicin resistant *E. coli* as the recipient. Transconjugants were selected on a Brain heart infusion agar (BHI agar) plate containing rifampicin (200 µg/mL). Transfer of plasmids, and co-dissemination of antibiotic resistance was confirmed by PCR, plasmid isolation, and antibiotic susceptibility testing for the transconjugants and recipient, as previously done for the donor.

Data Analysis

The data obtained in this research were analysed using descriptive statistics: plates, figures, percentages and tables using Microsoft word 2013, and the blood and stool was compared by using Chi-square at level of significance $p<0.05$ using SPSS.

RESULTS AND DISCUSSION

The main aim of this study was to investigate the antibiotics susceptibilities and plasmid prolife of *Salmonella typhi* isolated among patients attending Yobe State Specialist Hospital Damaturu.

Table 1 showed the numbers of samples collected for each of the specimen and the numbers of *S. Typhi* isolated from each of the specimen. A total number of 233 samples where collected out of which blood is 100(43%) and stool is 133(57%), 15 samples (57.1%) from blood samples confirmed the presence of *S. typhi* while 20 samples (42.9%) from stool showed growth of *S. typhi.* The results agreed with the findings of Melita (2011) and Kabir *et al*. (2007) who also reported high positive results of *Salmonella typhi* from blood samples. This study also agrees with the study of Okonko *et al*. (2010) where they reported less number of *Salmonella typhi* from stool samples than the blood samples.

Table 2.The multidrug resistance (MDR) pattern of 35 *Salmonella typhi* isolates found to be resistance to three and above antibiotics. The isolates where highly resistant was against tarivid (100%) less resistance was against pefloxacin (13.3%) in stool and on other Isolates where highly resistant was against chlorophenicol (60%) less resistance was against cefriaxone (20%) in blood.

Table 3 The total number of positive samples for extended beta lactamase production *Salmonella typhi* among the MDR isolates. Fourteen (14) *Salmonella typhi* isolates shows ESBL production; the Stool has the highest ESBL production with (57.1%) while least blood with (42.8%)

Table 4 The plasmid sizes and number of plasmids detected in *Salmonella typhi* isolate show by Agarose gel electrophoresis in this study, various plasmids of different molecular weight ranging from 2.4kb to 23.1kb were isolated *Salmonella typhi* from stool and Isolates harboured single to multiple (1-4) plasmids, Occurrence of plasmid was more in isolates from stool compared to those from blood Only one isolate isolated from blood harboured 3 plasmids.

TThe plasmid transfer by conjugation from the donor *Salmonella typhi* isolates to the *Escherichia coli* recipient show by agarose gel electrophoresis the donor transfer only one plasmid.

Thirty five 35 isolates tested positive for *S. typhi* comprising of 20 (57%) culture from blood and 15 (43%) culture from stool. The highest level of resistance was against tarvid (60%) follow by cefotaxime (51.4%) ceftazidine and chlorophenicol with (48.5%). Fourteen (14) *Salmonella typhi* isolates shows ESBL production; the stool has the highest ESBL producer with (57.1%) and blood has (42.8%). The PCR amplification of resistant genes were detected those that shows positive ESBL and *Salmonella* typhi harboured plasmids with sizes ranging from 2.4 to 23.1 kb. And Four of fourteen *S. typhi* ESBL-producing strains harboured 23 kb self-transmissible plasmid that was co-transferred with cefotaxime resistance to *Escherichia coli* transconjugants the bands were separated using agarose gel electrophoresis.

Table 1. *Salmonella Typhi* isolates from blood and stool sample

Specimen	Number of Samples (%)	Number of Positive for *S. typhi* (%)

Blood	100 (43)	20 (42.9)
Stool	133 (57)	15 (57.1)
Total	233 (100)	35 (100)

Significant at p<0.05 X^2=0.001

Table 2. Antibiotics susceptibility patterns and occurrence of Multi Drug resistant *Salmonella typhi* isolated from patients attending Yobe State Specialist Hospital Damaturu

Antimicrobial Agents (Concentrations)	**Symbols**	**Stool Isolates (%) (n = 15)**	**Blood Isolates (%) (n = 20)**	**Multi Drug Resistant Isolates Combined (%) (n = 35)**
Amoxicillin (10ug)	AM	10 (66.6)	5 (25)	15 (42.8)
Ceftazidine (30ug)	CAZ	8 (53.3)	7 (35)	17 (48.5)
Chloramphenicol(10ug)	CH	5 (33.5)	12 (60)	17 (48.5)
Cefotaxine (30ug)	CTX	7 (46.6)	11 (55)	18 (51.1)
Ciprofloxacin (30ug)	CPX	10 (66.6)	6 (30)	16 (45.7)
Augmentin (30ug)	AU	10 (66.6)	5 (25)	15 (42.8)
Gentamycin (10ug)	CN	9 (60)	7 (35)	16 (45.7)
Pefloxacin (30ug)	PEF	2 (13.3)	8 (40)	10 (28,5)
Tarvid (10ug)	OFX	15 (100)	6 (30)	21 (60)
Ceftriaxone (30ug)	CPO	9 (60)	4 (20)	13 (37.1)

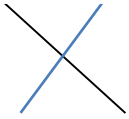
Table 3. Total number of extended beta lactamase producing *Salmonella typhi* isolated from patients attending state specialist hospital Damaturu

Source	**Number of Extended beta lactamase producing (%) n=14**
Blood	6 (42.8)
Stool	8 (57.1)
Total	14 (100)

Table 4. Plasmid sizes and number of plasmids detected in *Salmonella typhi* isolates

Isolate number	**Number of Plasmid**	**Estimated molecular sizes of Plasmid (Kb)**
1	2	20.5 & 7.8
2	2	20.5 & 7.8
3	3	19.8, 14.6, & 3.4
4	2	23.1, & 19.2
5	1	4.7
6	4	27.1, 12.3, 8.7, & 6.2
7	2	23.1, & 19.2
8	3	18.9, 14.6 & 3.4
9	2	19.2 & 6.6
10	3	23.1, 19.8 & 4.7
11	2	14.6 & 12.3
12	1	2.4
13	2	20.2 & 17.8
14	2	20.2 & 17.8

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 NC PC



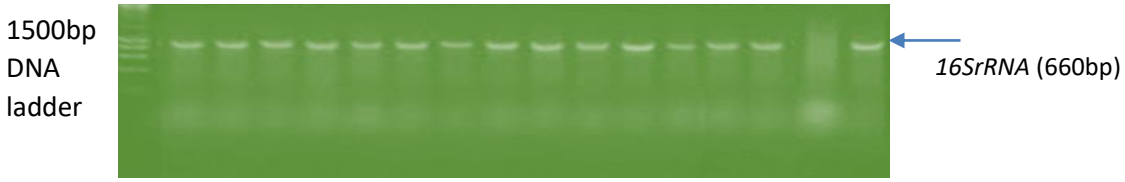


Plate 1. Agarose gel electrophoresis of the amplified *16SrRNA gene* Salmonella gene. Lane M represents 1500bp DNA molecular ladder, Lane PC represents Positive control; Lane NC represents Negative, Lane 1, 2 3 to Lane 14 represent the expression of the *16SrRNA* (660bp) gene for the Salmonella genus.

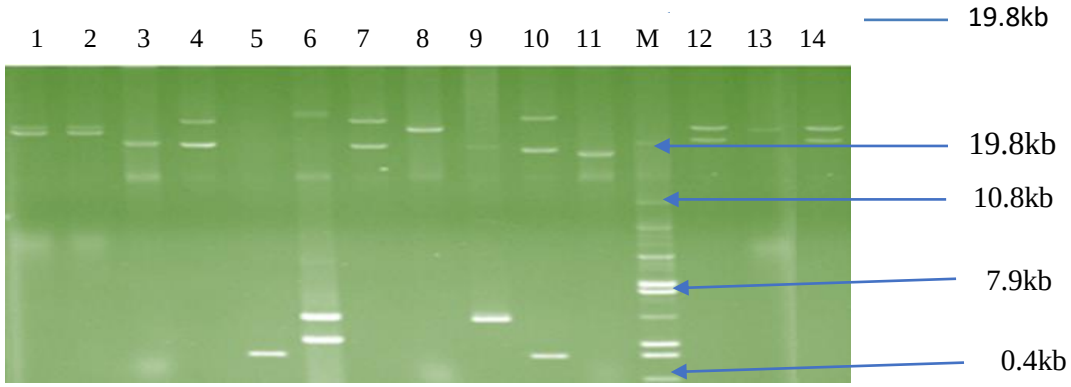


Plate 2: Agarose gel electrophoresis of the Plasmids of molecular weight of 0.4kb to 35.3kb detected in *Salmonella* isolates. Lane M represent Lambda DNA Hind III digest, Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14 represent the expression of the Hind III plasmid digested with their corresponding bands.

Table 5. Plasmid transfer by conjugation from the donor *Salmonella typhi* isolates to the *Escherichia coli* recipient

Donor *S. typhi* isolate		***E. coli* transconjugants**
Strain ID	**Plasmid profile, kb**	**Plasmid transferred, kb**
1	23, 7.8	23
4	23, 19.2	23
7	23, 19.2	23
10	23, 19.8 & 4.7	23

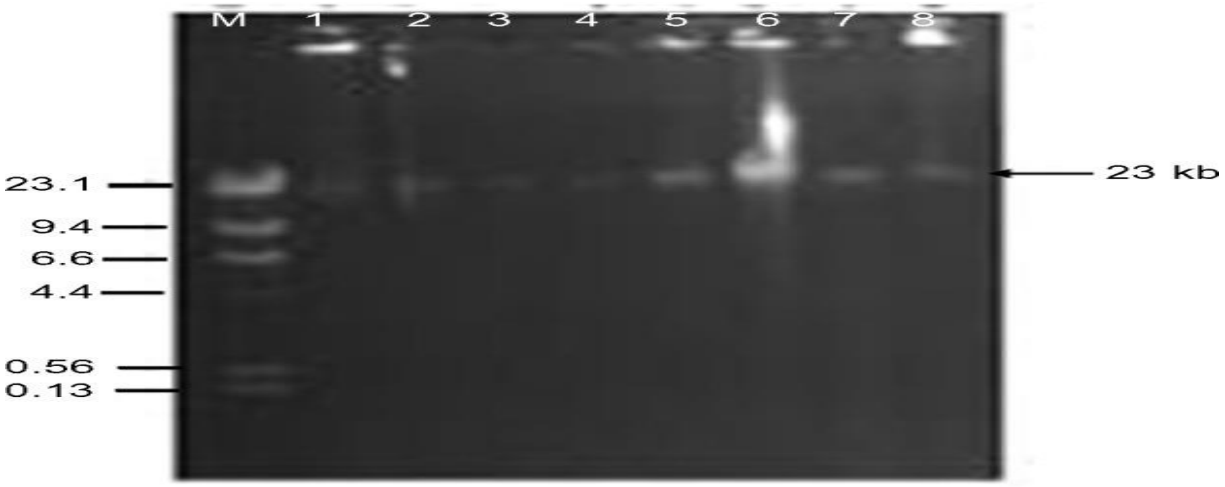


Plate 3. Plasmid transfer by conjugation from donor *Salmonella* typhi strains to recipient Escherichia coli j53-2. Lanes 1, 3, 5, and 7 are S. typhi donors Lag-003, -004, -007, and -010. Lanes 2, 4, 6, and 8 are E. coli j53-2 trans conjugants. Lane M represents Lamda DNA Hind III markers.

CONCLUSION

Salmonella infection remains a distressing public health concern worldwide**.** The findings of this study showed that ceftriaxone and pefloxacin are sensitive on *Salmonella typhi*. The laboratory culture of organism and antibiotic sensitivity testing should be encouraged in health institutions to avoid emergence of drugs resistance bacteria and effective and efficient management and control of disease

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Conflicts of Interest: The authors declare that they have no competing interest.

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